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Genetics

Mutations that alter the third cytoplasmic loop of the a-factor receptor lead to a constitutive and hypersensitive phenotype

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ABSTRACT **The *STE3* gene of *Saccharomyces cerevisiae* encodes a G protein-coupled receptor that is specific for the mating pheromone a-factor. The *ste3L194Q* mutation, which leads to the substitution of glutamine for leucine-194 within the third cytoplasmic loop of the receptor, resulted in a 20-fold increase in pheromone sensitivity and also caused partial constitutive activation of the response pathway. Moreover, other amino acid substitutions at the 194 position and several deletion mutations that collectively remove most of the third cytoplasmic loop resulted in hyperactive receptors. Therefore, we suggest that one role of the third cytoplasmic loop is to function as a negative regulatory domain involved in the maintenance of a nonsignaling state of the receptor. The constitutive activity and the pheromone hypersensitivity of *ste3L194Q* cells were recessive, suggesting that the wild-type receptor can antagonize the signal associated with the activated receptor. The *ste3Δ306* mutation, which results in truncation of most of the C-terminal domain of the receptor, led to a 20-fold increase in pheromone sensitivity, indicating that this domain also mediates negative regulation of the receptor. The *ste3L194Q* and *ste3Δ306* mutations appear to affect receptor activity independently, because the double mutant was associated with a 400-fold increase in pheromone sensitivity.**

The pheromone receptors of the yeast *Saccharomyces cerevisiae*, which permit communication between the **a** and α mating types, are structurally and functionally similar to the rhodopsin/adrenergic receptor family. First, the predicted amino acid sequences of the pheromone receptors imply that they contain seven membrane-spanning segments, followed by a relatively large cytoplasmic C-terminal domain (1–3). Second, the pheromone receptors couple to a heterotrimeric guanine nucleotide-binding protein (G protein) (4–6). The pheromone-activated receptors are believed to cause the exchange of bound GDP for GTP on the G_α subunit and the attendant release of the $G_{\beta\gamma}$ dimer. Free $G_{\beta\gamma}$ then causes the activation of downstream pathway components, ultimately leading to transcriptional induction of target genes and to arrest of the mitotic cell division cycle in the G_1 phase (6).

Mutational analysis of several members of the seven-transmembrane receptor family has revealed that they each exhibit a similar organization of functional domains (7). The transmembrane segments are thought to form a ligand-binding pocket (8–10), and the cytoplasmic loops that connect these transmembrane segments, especially the third cytoplasmic loop (see Fig. 2), are proposed to mediate coupling to specific G proteins (11, 12). The C-terminal cytoplasmic domain serves as a target for desensitization functions that negatively regulate receptor activity, and cells expressing C-terminally truncated receptors exhibit a hypersensitive response to stimulus (13–15). The third cytoplasmic loop may also function to negatively regulate receptor activity. This region of the β_2-adrenergic receptor is the target of a cAMP-dependent protein kinase that desensitizes the receptor, possibly by directly uncoupling it from the G protein (16). Moreover, for both the β_2- and α_{1B}-adrenergic receptors, this loop appears to constrain receptor activation and thereby prevent spontaneous receptor signaling in the absence of ligand (17–19). Whether either negative regulatory function ascribed to the third cytoplasmic loop is a general feature of the receptor family is not known.

We have carried out a mutational analysis of the third cytoplasmic loop of the yeast **a**-factor receptor. Deletions that remove part of the loop and many amino acid substitutions at a single site within the loop create receptors with both a constitutive and hypersensitive phenotype. We therefore suggest that one role of the third cytoplasmic loop is to function as a negative regulatory domain that ensures a nonsignaling resting state of the receptor in the absence of pheromone.

MATERIALS AND METHODS

Yeast Strains, Media, and Plasmids. All strains used were derived from SY1937 (*MATα STE3 ste2Δ mfa1Δ mfa2Δ::FUS1–lacZ FUS1::HIS3 ura3-52 leu2-3,112 ade1*) by standard transformation and gene replacement techniques (20, 21). YEP and synthetic media have been described (22).

pSL1596 is a pRS316 (23)-based plasmid that contains a 2.8-kb *Sal* I–*Sst* I fragment of the *STE3* locus (24). Substitution and deletion mutations of the proposed third cytoplasmic loop of *STE3* were created using pSL1596 and site-specific mutagenesis involving appropriate synthetic oligonucleotides. The mutations were confirmed by dideoxy sequencing using Sequenase (United States Biochemical). The oligonucleotides used to create the deletions also led to the insertion of one or two codons: deletion *ΔA* has an insertion of Val; *ΔB*, Arg; *ΔC*, Pro; *ΔE*, Asp-Ile; and *ΔF*, Asp-Ile. The *ste3Δ306* mutation is an in-frame deletion of the *Xmn* I–*Pst* I fragment of *STE3*, with the correct reading frame maintained by insertion of an *Sal* I linker. The result is the deletion of Lys-306 to Gly-469 and the addition of an Asn-Arg dipeptide.

Plasmid pSL2239 contains the *sst2Δ* allele and was used to create SY1955. The *sst2Δ* allele was created by the elimination of an *Nst* I–*Nsi* I fragment within the *SST2* gene (25). Plasmid pGCB contains *ste4Δ310-346* (26) and was used to create SY2466. pSL1469 was used to make *ste3Δ::URA3*, and pSL1448 was used to make an unmarked deletion, *ste3Δ*. Both plasmids delete the same region of the *STE3* locus, from an *Rsa* I site 417 bp upstream of the AUG to an *Sac* I site 111 bp downstream of the stop codon (3).

Substitution of various *STE3* alleles at the *STE3* locus was achieved by the one-step gene replacement technique (20) using constructs that contained *LEU2* sequences inserted ≈300 bp downstream of the *STE3* stop codon. These con-

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structs were used to transform *ste3Δ::URA3* derivatives of SY1937, SY1955, and SY2466. Plasmids pSL1601, pSL1641, pSL1645, and pSL1647 were used to substitute *STE3*, *ste3L194Q*, *ste3L194QΔ306*, and *ste3Δ306*, respectively, at the *STE3* locus, whereas plasmids pSL1683, pSL1713, pSL1715, and pSL1717 were used to substitute *GAL1* promoter-driven versions of the same *STE3* alleles. Substitution of *STE3* on the 3′ side of the *LYS2* locus on chromosome II was achieved by using plasmid pSL2105 and the two-step gene replacement procedure (20).

Halo and β-Galactosidase Assays. β-Galactosidase assays were performed as described (27). For halo assays, ≈5 × 10^4 exponential-phase cells were spread on the surface of a YEPD agar plate and tested for response to synthetic **a**-factor. The synthetic **a**-factor was diluted from a 1-μg/μl methanol solution into YEPD, and 2 μl was applied to the surface of the plate. Synthetic **a**-factor was kindly provided by Jeffrey Becker (28).

RESULTS

Many Alterations Within the Third Cytoplasmic Loop of STE3 Lead to Pheromone Hypersensitivity. Suppression of a nonsense mutation in the **a**-factor receptor structural gene (*ste3-1*) by a glutamine-inserting suppressor tRNA causes cells to become hypersensitive to **a**-factor (29, 30). This observation suggested that substitution of a glutamine for leucine at residue 194, the position affected by the nonsense mutation, would yield a receptor that conferred hypersensitivity to pheromone. We tested this possibility by using site-directed mutagenesis to create the *ste3L194Q* mutation. Cells producing the ste3L194Q receptor were about 20-fold more sensitive to pheromone than wild-type cells, as assessed by pheromone-mediated G_1 arrest or by induction of *FUS1–lacZ* (Fig. 1 *A* and *B*), a pheromone-inducible gene (31, 32). The *ste3L194Q* mutant was also more sensitive than a wild-type strain to pheromone-mediated arrest of cell division as measured by halo assay (Fig. 1*C*).

To learn whether the increased pheromone sensitivity of the *ste3L194Q* mutant was a specific property of that substitution or a general property of alterations in the third cytoplasmic loop, we created a larger family of mutations. First, site-directed mutagenesis was used to create a library of random mutations at codon 194. As assayed by induction of *FUS1–lacZ*, most substitutions resulted in a pheromone sensitivity that was greater than wild type, but none exhibited a more extreme phenotype than that caused by the glutamine substitution. A subset of the mutations, chosen because they conferred a range of sensitivities, were sequenced to ascertain the amino acid substitution. The relative levels of *FUS1–lacZ* induction associated with particular substitutions were Leu = Phe < Ala < Arg = Gln (data not shown). A few mutations conferred a nonresponsive phenotype, but these all proved to be nonsense mutations. Second, a series of deletions that collectively removed the sequences encoding the third cytoplasmic loop were constructed (Fig. 2*A*). Most of the deletions, *ste3ΔB–ste3ΔE*, did not prevent response to pheromone, but rather conferred increased sensitivity to pheromone (Fig. 2*B*). We conclude that the sequences directly surrounding and including Leu-194 are not essential for signaling. Instead, the hypersensitivity associated with several deletion mutations suggests a negative regulatory function for this region of the third cytoplasmic loop.

Cells expressing receptors with deletions that occur near the proposed N- and C-terminal portions of the loop, *ste3ΔA* and *ste3ΔF*, were impaired for receptor signaling (Fig. 2*B*). These mutant receptors are potentially defective in G-protein coupling following pheromone interaction, because similar regions of the β_2-adrenergic receptor appear to be important for productive interaction with $G_{\alpha s}$ (16, 33, 34). However, initial experiments suggest that these mutant receptors are at least partially defective for transit from the endoplasmic reticulum to the cell surface (data not shown), which may contribute to their impaired function or reflect the loss of normal receptor structure.

The pheromone receptors are metabolically dynamic. They are transported via the secretory pathway to the cell surface, where they reside only transiently before being internalized and delivered to the vacuole for degradation (35). In addition to this ligand-independent endocytosis, the receptors are also subject to ligand-triggered endocytosis, which may be mechanistically distinct (35). To examine the effect of the *ste3L194Q* mutation on receptor metabolism, we measured the half-life of the ste3L194Q receptor by a pulse–chase protocol using [^{35}S]methionine to label the receptor. As shown by the initial time point in Fig. 3, ste3L194Q is synthesized at a slightly greater rate (≈1.5×) than the wild-type receptor. This difference can be attributed to the facts that *ste3L194Q* cells show a higher basal expression of pheromone-inducible genes (see below) and that *STE3* tran-

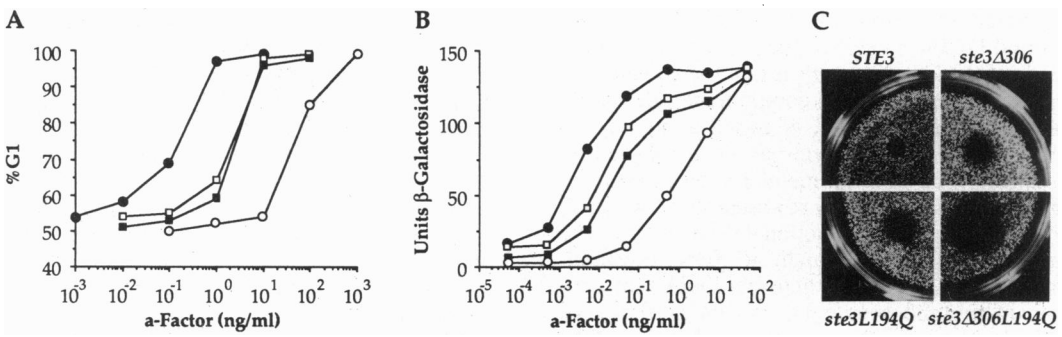

FIG. 1. Pheromone sensitivity of *ste3* mutants. Pheromone response was evaluated for a set of isogenic derivatives of strain SY1937 that differed only at the *STE3* locus. (*A*) *STE3* (○), *ste3L194Q* (□), *ste3Δ306* (■), and *ste3L194QΔ306* (●) strains were grown to a density of ≈2 × 10^7 cells per ml in YEPD medium, treated with the indicated concentrations of synthetic **a**-factor for 3 hr, fixed by mixing 1:1 with 0.15 M NaCl/3.7% formaldehyde, and examined by phase-contrast microscopy. Unbudded cells and cells with projections were considered to be in the G_1 phase of the cell cycle. At least 300 cells were observed for each strain at a given pheromone concentration. The results of a single experiment are shown. The variability observed for independent experiments corresponds to ±2% of the reported values. (*B*) *STE3* (○), *ste3L194Q* (□), *ste3Δ306* (■), and *ste3L194QΔ306* (●) strains were grown to exponential phase in YEPD and exposed to various concentrations of synthetic **a**-factor for 1 hr. Cells were subsequently prepared and assayed for β-galactosidase activity. The relative levels of β-galactosidase activity provide a measure of the pheromone induction of *FUS1–lacZ*. The results of a single experiment are shown. The variability observed for independent experiments corresponds to approximately ±10% of the reported values. (*C*) Response to pheromone was measured as a zone of growth inhibition, or halo. The photograph is a composite of four different assays, one for each strain indicated. The halo assays were performed with ≈179 ng of synthetic **a**-factor.

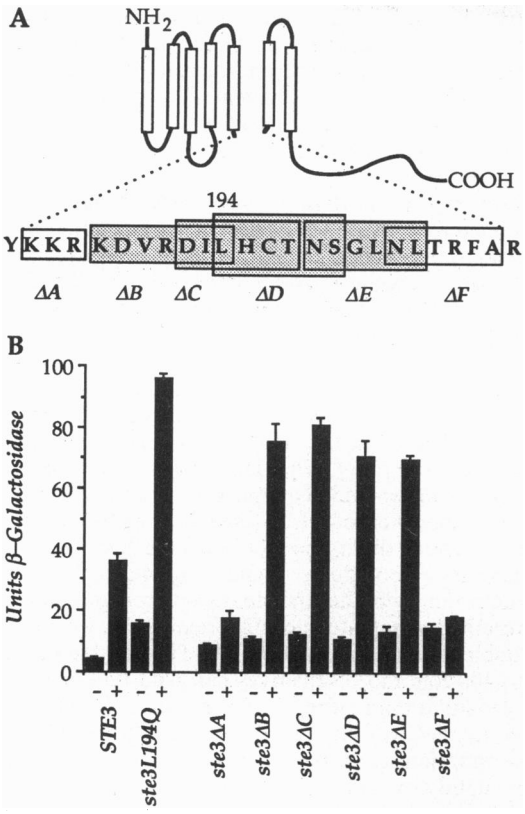

FIG. 2. *FUS1–lacZ* expression by strains carrying mutations that alter the third cytoplasmic loop of the a-factor receptor. (*A*) The amino acid sequence predicted to form the third cytoplasmic loop, from Tyr-185 to Arg-208, is presented. Deletion mutants *ste3ΔA–ste3ΔF* span this domain. Boxes representing Δ*B*, the Δ*C*, Δ*D*, and Δ*E* mutations are shaded to indicate that the mutant receptors exhibit a similar degree of sensitivity (see *B*). (*B*) SY2011 (*MATα ste3Δ ste2Δ mfa1Δ mfa2Δ::FUS1–lacZ*) was transformed with a single-copy plasmid containing the indicated *STE3* alleles. Transformants were grown in synthetic medium to select for plasmid maintenance. Exponential cultures were diluted with an equal volume of 2× YEPD and grown for 2 hr. Each culture was then divided into two equal volumes, one of which was exposed to synthetic a-factor (0.25 ng/ml, +) for 1 hr. Then cells were prepared and assayed for β-galactosidase activity. The results of three experiments were averaged; error bars indicate 1 SD.

scription is pheromone-inducible (36). The higher levels of mutant receptor cannot account for the hypersensitivity because overproduction of the wild-type receptor does not lead to increased pheromone sensitivity (data not shown). Similarly, overexpression of the α-factor receptor does not lead to increased pheromone sensitivity, suggesting that the pheromone receptors are not limiting for signaling (14, 15). Fig. 3 also shows that both wild-type and ste3L194Q receptor

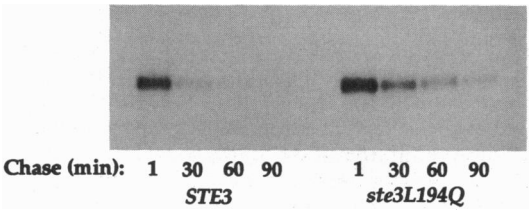

FIG. 3. Turnover of STE3 and ste3L194Q. *STE3* and *ste3L194Q* derivatives of SY1937 were labeled for 10 min with [^{35}S]methionine as described (35). Samples were taken 1, 30, 60, and 90 min after initiation of the chase, extracts were prepared, and receptor protein was precipitated with antiserum raised against the C-terminal domain of STE3 (35). Immunoprecipitates were subjected to SDS/PAGE and STE3 was visualized by autoradiography.

were unstable, exhibiting a half-life of 20–30 min. This finding implies that ligand-independent endocytosis of the ste3-L194Q receptor is normal. The ste3L194Q receptor is also normal for pheromone-triggered endocytosis (data not shown). Thus, we conclude that the phenotype associated with the ste3L194Q receptor reflects a change in its intrinsic signaling properties rather than a change in its metabolism.

Alterations of the Third Cytoplasmic Loop Lead to Constitutive Activation of the Pheromone Response Pathway. To test whether the hypersensitive *STE3* mutants also exhibited a constitutive phenotype, we examined the basal expression of *FUS1–lacZ* in the absence of pheromone. The strain used was deleted for the a-factor structural genes and the α-factor-receptor structural gene to preclude autocrine stimulation. In the absence of pheromone, *ste3L194Q* cells displayed a basal signaling activity that was significantly greater than that of *STE3* cells (Fig. 2*B*). Receptors with deletions within the third cytoplasmic loop also displayed an increased basal signal (Fig. 2*B*).

To facilitate physiological and genetic analysis of the constitutive signal associated with various receptors, the receptor structural genes were placed under the control of the *GAL1* promoter (37). The *GAL1* promoter leads to a 10- to 20-fold overproduction of the receptor without perturbing its metabolism (35) and normalizes the expression of STE3 and ste3L194Q proteins (data not shown). An increase in *FUS1–lacZ* expression was apparent when the ste3L194Q receptor was transiently produced by the *GAL1* promoter-driven construction, but no increase in *FUS1–lacZ* expression was observed upon similar production of the wild-type receptor (Fig. 4). Deletion of *STE4* (G_β) blocked the constitutive signal (Fig. 4). Conversely, deletion of *SST2*, which is required for desensitization to pheromone, possibly through regulation of the G protein (10), greatly enhanced the constitutive signal, as evidenced by increased *FUS1–lacZ* expression (Table 1) and by cell cycle arrest in the G_1 phase (data not shown). Thus, the constitutive signal of *ste3L194Q* cells is *STE4*-dependent and subject to *SST2*-mediated desensitization.

The Pheromone Hypersensitivity and Constitutive Signaling Phenotypes of *ste3L194Q* Mutants Are Recessive. Characterization of the constitutive signal of *ste3L194Q* mutants suggested that this altered form of the receptor can promote

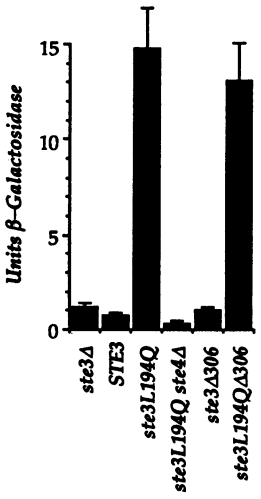

FIG. 4. *FUS1–lacZ* expression by *ste3L194Q* mutants. Cells containing various chromosomal *GAL1–STE3* constructs were grown to exponential phase in YEP/raffinose. Expression of the receptor was then induced by addition of galactose (2%, wt/vol). After 6 hr of growth in the galactose-containing medium, cells were isolated and assayed for β-galactosidase activity. All strains were isogenic, differing only by the indicated alleles. The results of three experiments were averaged; error bars indicate 1 SD.

Table 1. Constitutive expression of *FUS1–lacZ* in various genetic backgrounds

Strain	*FUS1–lacZ* expression
STE3	1.2 ± 0.2
STE3 sst2Δ	12.6 ± 1.0
ste3L194Q	14.8 ± 2.1
ste3L194Q sst2Δ	118.4 ± 6.3
STE3 ste4Δ310-346	11.1 ± 2.5
ste3Δste4Δ310-346	26.8 ± 3.3

All strains were isogenic, differing only by the indicated alleles. *STE3* and *ste3L194Q* were under control of the *GAL1* promoter and integrated at the *STE3* locus. Cells were grown and induced for expression of the receptor genes as described in the legend to Fig. 4. The results of three experiments were averaged; the error indicates 1 SD.

guanine nucleotide exchange in the absence of pheromone. Because this phenotype appears to represent a gain of function, we expected that the constitutive property of *ste3L194Q* mutants would be dominant. Surprisingly, the basal expression of *FUS1–lacZ* was low in cells that expressed both *ste3L194Q* and *STE3* (Fig. 5*A*). That is, the constitutive phenotype was recessive. Similarly, the hypersensitivity of *ste3L194Q* cells to pheromone was recessive (Fig. 5*B*). These results suggest that the wild-type receptor can regulate the pronounced signaling activity associated with the hyperactive receptor. Since the constitutive signal of the *ste3L194Q* mutant was recessive, the wild-type receptor, when unoccupied by ligand, may provide this regulatory function. Several different mechanisms could account for this observation. We favor the possibility that the pheromone-unoccupied form of STE3 can interact with and influence the signaling activity of downstream components of the response pathway. In agreement with this notion, we have observed

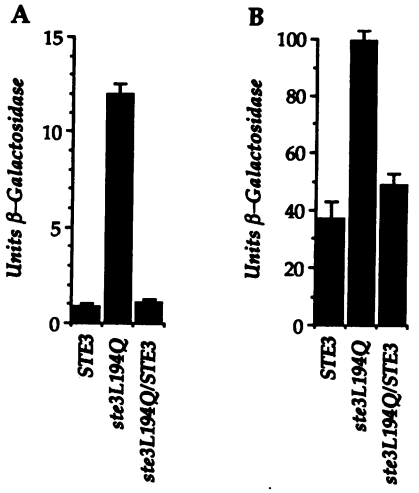

FIG. 5. Test for dominance of the constitutive and hypersensitive phenotypes associated with *ste3L194Q*. (*A*) Constitutive phenotype. Transcription of the *STE3* alleles was under the control of the inducible *GAL1* promoter. *GAL1–ste3L194Q* was integrated at the *STE3* locus. The *GAL1–STE3* allele was integrated 3′ to the *LYS2* locus on chromosome II. All strains contained *FUS1–lacZ* and were isogenic except at the indicated loci. Cells were induced for receptor expression and subsequently analyzed for β-galactosidase activity as described in the legend to Fig. 4. The results of three experiments were averaged; error bars indicate 1 SD. (*B*) Hypersensitive phenotype. Transcription of the *STE3* alleles was under control of the natural promoter. *ste3L194Q* was integrated at the *STE3* locus, and *STE3* was integrated 3′ to the *LYS2* locus on chromosome II. Cells were grown to exponential phase in YEPD and then exposed to synthetic a-factor (0.25 ng/ml) for 1 hr. The cells were prepared and assayed for β-galactosidase activity. The results of three experiments were averaged; error bars indicate 1 SD.

that expression of *STE3* attenuates the constitutive signal associated with an alteration of G_β, *ste4Δ310-346* (ref. 26 and Table 1). Furthermore, the pheromone hypersensitivity associated with C-terminal truncated forms of the α-factor receptor is recessive (14, 15), suggesting that the pheromone-unoccupied form of STE2 can also serve a regulatory function.

C-Terminal Truncation of ste3L194Q Further Increases Pheromone Responsiveness. The C-terminal domain of some members of the rhodopsin/adrenergic receptor family serves as a negative regulatory domain (7, 13, 38). To determine whether the C-terminal domain contributes to negative regulation of the a-factor receptor, the phenotype of cells carrying the *ste3Δ306* mutation, which truncates most of the C-terminal domain, was examined. Cells producing this altered form of the receptor were hypersensitive to pheromone and showed a response that was similar to that of cells expressing *ste3L194Q* (Fig. 1). However, unlike the *ste3-L194Q* mutants, *ste3Δ306* mutants did not show substantially elevated expression of *FUS1–lacZ* in the absence of pheromone, indicating that removal of the C-terminal domain does not generate a constitutive signal (Fig. 4).

To determine whether the ste3L194Q receptor was subject to C-terminally mediated negative regulation, we constructed the double mutant *ste3L194QΔ306*. This double mutant was 200- to 400-fold more sensitive than wild type and ≈20-fold more sensitive than either single mutant (Fig. 1). The exaggerated hypersensitivity associated with the *ste3L194QΔ306* double mutation suggests that the two receptor alterations act independently to confer pheromone hypersensitivity. In contrast, the *ste3Δ306* mutation did not enhance the ligand-independent expression of *FUS1–lacZ* associated with *ste3L194Q* mutants (Fig. 4), implying that the constitutive signal is not attenuated by C-terminally mediated regulation.

DISCUSSION

Our mutational analysis of the third cytoplasmic loop of the a-factor receptor has revealed that one role of this loop is to mediate negative regulation of receptor activity and thereby maintain the receptor in an inactive state until it is stimulated by an agonist. A number of different amino acid substitutions for Leu-194 of STE3, of which ste3L194Q is the archetype, caused a substantial increase in sensitivity to pheromone. Moreover, several deletion derivatives also resulted in pheromone hypersensitivity. Strikingly, these alterations also led to partial activation of the pheromone response pathway, even in the absence of ligand. Finally, although *ste3L194Q* and *ste3Δ306* cells exhibit the same sensitivity to standard a-factor, preliminary studies suggest that *ste3L194Q* cells are considerably more sensitive to partially active forms of the pheromone (G. Caldwell, F. Naider, and J. Becker, personal communication). Together, these findings indicate that loss of normal structure in the third cytoplasmic loop results in a receptor with a greater probability of generating a signal, both in the presence and in the absence of pheromone.

Analysis of the adrenergic receptors has suggested two mechanisms by which the third cytoplasmic loop could participate in negative regulation of receptor activity. First, as noted in the Introduction, phosphorylation of a particular serine residue in the third cytoplasmic loop of the β_2-adrenergic receptor leads to desensitization (16, 39). β_2-Adrenergic receptor mutants in which a nonphosphorylatable residue replaced this serine were defective in desensitization to agonist but did not exhibit a constitutive phenotype (40). Second, a region of the third cytoplasmic loop of both the β_2-adrenergic and the α_{1B}-adrenergic receptors appears to constrain receptor activation and maintain the receptor in a nonsignaling mode unless bound by agonist (19). This conclusion followed from the identification of constitutive forms

of the adrenergic receptors (17–19). For example, all amino acid substitutions at a single site of the α_{1B}-adrenergic receptor led to agonist-independent coupling to the G protein as well as an increased affinity for agonist (18). Thus, the properties associated with the loss of normal structure in the third cytoplasmic loop of the mutant adrenergic receptors mimic those of the agonist-activated wild-type receptor. Computer simulations imply that these mutant receptors isomerize to an active state more readily than do wild-type receptors (19). That is, increased G-protein coupling is a secondary consequence of the change in the isomerization properties of the mutant receptor.

Our finding that many alterations in the third cytoplasmic loop of the a-factor receptor led to a hypersensitive and constitutive phenotype parallels the genetic findings made for the α_{1B}-adrenergic receptor. At present, we cannot exclude the possibility that the primary affect of the alterations is to increase coupling to the G protein, but the genetic parallels to the α_{1B}-adrenergic receptor suggest that this region of the a-factor receptor also functions to constrain receptor activation. Since the a-factor receptor and the adrenergic receptors are unrelated in primary sequence, our observations suggest that a constraining role for the third cytoplasmic loop may be a common property of this receptor family.

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